Chapter 6
Neuroprotection Versus Neurotoxicity

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Abstract Several original concepts concerning microglia have changed in the last decade. Ramified microglia are no longer seen to be "resting" cells and it also is very obvious today that microglia responses are by no means stereotypic, but manifold and targeted. Moreover, there is good evidence that microglia are not only important in brain pathology, but that they also play important roles in the healthy brain. One long-standing aspect of microglia biology, however, was never questioned: their involvement in brain disease. Based on morphological changes (retraction of processes and amoeboid shape) that inevitably occur in these cells in case of damage to the central nervous system, microglia in the diseased brain were called "activated". Because "activated" microglia were always found in direct neighbourhood to dead or dying neurons, and since it is known now for more than 20 years that cultured microglia release numerous factors that are able to kill neurons, microglia "activation" was often seen as neurotoxic. From an evolutionary point of view, however, it is difficult to understand why an important, mostly post-mitotic and highly vulnerable organ like the brain would host numerous toxic cells. How microglia can protect the nervous tissue and what might go awry when microglia turn neurotoxic will be discussed in this chapter.

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Bullet Points

- Microglia are the myeloid cells of the brain and play an important role in all brain diseases.
- Because "activated" microglia are always found in direct neighbourhood to dead or dying neurons, and since microglia in culture are able to kill neurons, microglia "activation" in the brain is often seen as neurotoxic response.
- The inhibition or deletion of microglia, however, often results in a worsening of disease symptoms, indicating that microglia activity can be protective in the brain.
- How microglia can protect the nervous tissue and what might go awry when microglia turn neurotoxic will be discussed in this chapter.

6.1   Microglia Physiology and Function

Microglia research truly started in 1932 by Pío del Río-Hortega, who introduced microglia (and numerous, even today, still valid postulates concerning these cells) in a chapter for "Cytology and Cellular Pathology of the Nervous System", a milestone in neuroscience that was edited by Wilder Penfield (del Rio-Hortega 1932); see for an excellent review about the history of microglia research (Kettenmann et al. 2011) and Chap. 2.

Despite del Río-Hortega's work and the results of many others that continued the study of microglia after him, these cells have been widely neglected by neuroscientists to a point that even in leading textbooks of neuropathology of the early 90s their mere existence was doubted (see for details Graeber 2010). This ignorance most likely was due to the fact that the original method for staining microglia was tedious and highly variable. The 90s of the last century saw technological progress in microglia research. The introduction of microglia cultures, more easy and reliable staining techniques (antibodies, lectins), and the "green microglia" mouse (CX3CR1-EGFP strain introduced by Steffen Jung (2000)) made these cells more accessible for investigators. As a result, microglia research has intensified enormously in the last decade. Many surprising findings about microglia have been published and recent results have begun to provide convincing answers to the most basic questions in microglial biology, namely the origin of these cells or the issue whether or not microglia are replaced by peripheral cells throughout the lifetime of an individual (Ajami et al. 2007; Ginhoux et al. 2010; Kierdorf et al. 2013). Microglia in the healthy brain are characterized by a so-called ramified morphology, small cell bodies, and fine heavily branched processes. Ramified microglia are sessile cells, meaning their cell bodies do not move, which is in contrast to their processes that display the most pronounced motility yet described in the mammalian brain. This process movement is currently discussed to be part of the surveillance function, since in the direct vicinity of microglia other cellular elements are regularly touched by these cells (for further reading, see Chap. 4). Ramified microglia

are more or less evenly distributed throughout the brain and claim their own "territory". It is therefore estimated that due to their process motility the brain's parenchyma is "completely investigated" every few hours (Davalos et al. 2005; Nimmerjahn et al. 2005; Raivich 2005). In the last few years it was found that especially active synapses attract microglia process "interactions" and a role for these cells in regulating synaptic function is currently discussed elsewhere (Bechade et al. 2013; Miyamoto et al. 2013) (For further details, see Chap. 9). However, despite the fact that microglia research has enormously intensified and that microglia have been the subject of numerous recent and excellent reviews, we still know very little about their physiology and the function of ramified microglia in the healthy brain. As such, these cells remain among the most mysterious cells of the brain (Hanisch and Kettenmann 2007; Colton 2009; Ransohoff and Perry 2009; Yong and Rivest 2009; Graeber 2010; Parkhurst and Gan 2010; Ransohoff and Cardona 2010; Prinz and Mildner 2011; Tremblay et al. 2011; Aguzzi et al. 2013; Hanisch 2013; Hellwig et al. 2013; Kettenmann et al. 2013; Miyamoto et al. 2013; Sierra et al. 2013).

More is known about the function of microglia in brain disease. However, simplified concepts about their "activation" (see next paragraph) and the experimental difficulty to distinguish microglia from peripheral myeloid cells that often enter the diseased brain (Ransohoff and Cardona 2010; Prinz and Mildner 2011; Hellwig et al. 2013; London et al. 2013) have yet hampered the development of a clear view about whether or not these cells contribute to either initiation, course, or end of a given brain disease. Here we will discuss current knowledge about the role of microglia in brain disease with particular emphasis about microglia in neuroprotection or neurotoxicity.

6.2 Microglial Activation and Neuroinflammation Defined: Surveillance and Response

Almost any type of brain injury or disease inevitably leads to a fast morphological response of microglia. Ramified microglia retract their fine processes and acquire a morphology which resembles that of phagocytic macrophages, a transition that was originally described by del Río-Hortega. This morphological reaction often is correlated with a migratory behaviour and/or proliferation of the cells. Because the morphological complexity of this transition is reduced (basically only one morphological phenotype is the result of this process), it was originally defined as a stereotypic and graded process (Kreutzberg 1996; Streit 2002; van Rossum and Hanisch 2004; Hanisch and Kettenmann 2007). Based on these morphological data it was widely assumed that ramified microglia in the healthy brain would be inactive or resting and that the morphological transition of microglia in response to neuronal injury sets these cells into action, leading to describe this process as "microglia activation". Moreover, because the morphological transition appears to be a stereotypic process, it was more or less believed that "activated" microglia would respond in a stereotypic way with limited response variability. This general and simple

concept of microglia "activation" is not valid anymore (see for recent reviews: Hanisch and Kettenmann 2007; Colton 2009; Ransohoff and Perry 2009; Yong and Rivest 2009; Graeber 2010; Parkhurst and Gan 2010; Ransohoff and Cardona 2010; Kettenmann et al. 2011; Prinz and Mildner 2011; Tremblay et al. 2011; Aguzzi et al. 2013; Hanisch 2013; Hellwig et al. 2013; Kierdorf et al. 2013; London et al. 2013; Sierra et al. 2013). Not only is it clear today that ramified microglia are by no means resting cells (see above), it is also apparent now that microglia respond with a variety of different reactions by integrating multifarious inputs (Hanisch and Kettenmann 2007; Colton 2009; Ransohoff and Perry 2009; Yong and Rivest 2009; Graeber 2010; Parkhurst and Gan 2010; Ransohoff and Cardona 2010; Kettenmann et al. 2011, 2013; Prinz and Mildner 2011; Tremblay et al. 2011; Aguzzi et al. 2013; Hanisch 2013; Hellwig et al. 2013; Kierdorf et al. 2013; London et al. 2013; Sierra et al. 2013). In line with this, microglia responses are not inevitably neurotoxic as often believed. Various neuroprotective effects of "activated" microglia have been demonstrated recently in vivo (Boillee et al. 2006; Turrin and Rivest 2006; El Khoury et al. 2007; Lalancette-Hebert et al. 2007; Lambertsen et al. 2009). Conversely, neurotoxicity might occur in case of overshooting an uncontrolled response of microglia (van Rossum and Hanisch 2004; Cardona et al. 2006, and many others) or when microglia function is impaired (Boillee et al. 2006; Streit 2006; Neumann and Takahashi 2007). Moreover, microglia function is not only important in neurodegenerative and neuroinflammatory diseases, but there is accumulating evidence that immune malfunction is also involved in psychiatric diseases like schizophrenia and mood disorders. The control of microglia function has therefore recently become of interest in biological psychiatry (see for review: Muller and Schwarz 2007; Bernstein et al. 2009; Beumer et al. 2012; Blank and Prinz 2013; Stertz et al. 2013). Thus, microglia are currently considered to be involved in a wide range of brain disorders.

As described above, microglia function has long been correlated to morphological criteria. Ramified microglia in the healthy brain were described as "resting", whereas more amoeboid microglia in the diseased brain were thought to be "activated". An overwhelming body of evidence from the last decade has convincingly shown that this simple discrimination does not hold true. Since there are no inactive microglia (the moniker "never resting microglia" is quite popular among microglia-minded scientists at the moment (Karperien et al. 2013)), the terms microglia "activation" or "activated" microglia are misleading. Ramified microglia are clearly surveillance cells that most likely execute numerous functions in the healthy brain. These cells have been referred to as "cops on the beat" (Raivich 2005), "constant gardeners" (Hughes 2012), "industrious housekeepers" (Streit and Xue 2009) or "garbage men" (Kettenmann 2007). However, even these descriptions most likely do not reflect the whole functional spectrum of ramified microglia in the healthy brain. The various functions of microglia are controlled not only by a whole plethora of receptors for exogenous signals (Biber et al. 2007; Hanisch and Kettenmann 2007; Kettenmann 2007; Ransohoff and Perry 2009; Ransohoff and Cardona 2010; Kettenmann et al. 2011; Domercq et al. 2013), but also endogenously by subsets of transcription factors (Kierdorf and Prinz 2013). Microglia action may or may not

involve major morphological transitions (Vinet et al. 2012; Karperien et al. 2013) and the roles of amoeboid microglia are manifold (Kettenmann et al. 2011). Microglia merely adjust their current activity in response to changes in their direct vicinity, or as reactions to endogenous changes, such as aging (Kierdorf and Prinz 2013; Wong 2013). As such, the operational definition of microglial activation is a broad concept that only refers to the general process of altering either the physical characteristics, activity, or function of microglia. Given the variety of activation phenotypes possible, the term activation only notes that a cellular change occurred, providing little to no information about cellular function.

Consistent with this premise, simple morphological criteria (ramified versus amoeboid) are also poor indicators of microglial function. For example, the presence of amoeboid microglia near dead (or dying) neurons has often been interpreted as a proof of a neurotoxic microglia response (Hellwig et al. 2013). Based on our current knowledge about microglia, such morphological correlation does not provide any information about causality or function of these cells. It is thus clear that without (i) a detailed analysis of the causal relationship between microglial function and neuronal fate, and (ii) a thorough understanding of all aspects of the microglia response in a given disease (model), it is difficult to fully appreciate what microglia are doing in the brain. New mouse models that allow a more targeted analysis of microglia in vivo may be helpful here (Mizutani et al. 2012; Varvel et al. 2012; Neumann and Wekerle 2013; Wolf et al. 2013). Whether or not a more sophisticated, fractal analysis of microglia morphology will be useful as predictor of microglia function is discussed elsewhere (Karperien et al. 2013).

6.3 Microglia in Central Nervous System Disease and Neuron Damage

Hallmark studies in the 1980s analysing postmortem tissue samples from Parkinson's disease (PD) (McGeer et al. 1988) and Alzheimer's disease (AD) patients (McGeer et al. 1987, 1988) were the first to suggest that microglia positive for human leucocyte antigen (HLA) (a surface receptor complex involved in antigen presentation) were linked to active neurodegeneration (McGeer et al. 1988), which marked the beginning of the field of research dedicated to understanding the involvement of microglia in central nervous system (CNS) diseases. Since then, microglia have been identified as critical for the maintenance of normal, healthy CNS physiology and repair, in addition to being implicated as instigating actors of progressive neuronal damage in neurodegenerative disorders/CNS damage (Perry et al. 2010), including traumatic brain injury (Woodcock and Morganti-Kossmann 2013), AD (Naert and Rivest 2011), Amyotrophic Lateral Sclerosis (ALS) (Phani et al. 2012), Multiple Sclerosis (Doring and Yong 2011), Huntington's disease (Moller 2010), and PD (German et al. 2011).

Most information linking microglia to human neurodegenerative disease/CNS damage is correlative, demonstrating changes in morphology and elevation of

markers in the damaged brain regions, where heightened levels of pro-inflammatory molecules are often localized to microglia. PD, a movement disorder that is the second most prevalent neurodegenerative disease (Dorsey et al. 2007), is a classic example of how the microglial response is intricately linked to progressive neuron damage. PD is characterized by the progressive and selective degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) (Kish et al. 1992). Importantly, neuroinflammation is present, as pro-inflammatory cytokine levels are elevated in the SNpc of PD patients (Reale et al. 2009; German et al. 2011; Panaro and Cianciulli 2012). Postmortem tissue analysis also reveals elevation of HLA-DR positive microglia in the SNpc of PD patients (McGeer et al. 1988; Imamura et al. 2003; Orr et al. 2005), confirming a phenotypic change in microglia in regions of ongoing neuronal damage. Interestingly, microglial markers are also elevated in other brain regions implicated in PD outside of the SNpc (Imamura et al. 2003; Gerhard et al. 2006), consistent with the fact that despite hallmark DA neuron loss, PD neuropathology extends to many brain regions. For example, PD patients have shown significantly higher numbers of Major Histocompatibility Complex (MHC) class II-positive microglia in the hippocampus (HC), transentorhinal cortex, cingulate cortex, and temporal cortex, regions outside of the SN with reported neurite damage in PD (Imamura et al. 2003). MHC class II is crucial for antigen presentation, its role in PD however remains undetermined. It is known that MHC class II microglia in the SNpc of PD patient tissue are also positive for tumour necrosis factor α (TNFα) and interleukin 6 (IL-6) (Imamura et al. 2003), demonstrating that these cells are at least one source of CNS cytokines. Positron emission tomography (PET) imaging with the radiolabeled ligand ([11C](R)-PK11195) that binds the peripheral benzodiazepine receptor which in the brain is predominantly expressed by "activated" microglia confirms the presence of "activated" microglia in the SNpc of living PD patients (Gerhard et al. 2006), where increased levels of [(11)C](R)-PK11195 binding is associated with the loss of nigrostriatal DA neuron terminal function and disease severity (Ouchi et al. 2005). PET imaging also demonstrates that PK11195 binding is elevated in the SNpc early in the disease process of both PD and Lewy body dementia (PD-related disease) patients (Iannaccone et al. 2013), supporting that microglia are active throughout the disease process. The premise of a pro-inflammatory phenotype in microglial for AD is also supported by analysis of postmortem brains from AD patients (McGeer et al. 1987; Rogers et al. 1988), where the microglial response precedes neutrophil damage (Cagnin et al. 2001), suggesting a potential causal role. Together, evidence points to neuroimmune perturbation and microglial response in PD, AD, and in fact many human CNS diseases (Smith et al. 2012). Yet these important, but descriptive studies provide little functional data about causal relationships.

The use of animal models of CNS disease provides the experimental work implicating microglia as a culpable source of chronic cytokines and reactive oxygen species (ROS) that are toxic to neurons. As innate immune cells, microglia are capable of upregulating an entire battery of compounds identified as toxic to neurons (Phani et al. 2012), inducing TNFα (Harms et al. 2012; Abo-Ouf et al. 2013), inducible

nitric oxide synthase (iNOS) (production of nitric oxide) (Brown 2007), NADH oxidase 2 (NOX2) (production of superoxide) (Brown 2007; Sorce and Krause 2009), cyclo oxygenase 2 (COX2) (production of prostaglandin PGE_2) (Jiang et al. 2011), interleukin 1β (IL-1β) (Turola et al. 2012; Ye et al. 2013), and interferon γ (INFγ) (Mount et al. 2007). While not all pro-inflammatory factors are neurotoxic in all disease models and brain regions (Sriram and O'Callaghan 2007; Nadeau et al. 2011), multiple neurodegenerative disease models are benefited by an anti-inflammatory approach (Choi et al. 2013; Kay and Palmer 2013; Ramsey and Tansey 2014; Tabas and Glass 2013). For example, neuronal damage in a PD mouse model is significantly reduced in the SNpc of mutant mice with deficient production of pro-inflammatory factors, such as superoxide (Wu et al. 2003; Zhang et al. 2004), prostaglandins (Feng et al. 2002; Teismann et al. 2003), and TNFα (Sriram et al. 2002). Importantly, several studies have documented that microglia are a source of these potentially neurotoxic molecules in human neuron damage and neurodegenerative disease (Phani et al. 2012; Smith et al. 2012; Woodcock and Morganti-Kossmann 2013).

More recent and convincing evidence employs in vivo studies and myeloid cell-specific gene deletion (which includes microglia, neutrophils, monocytes, other macrophages, and other non-neuronal cells) in CNS disease/damage models to reveal that only certain components of the microglial response can drive neuronal damage. The inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (Ikkβ) is a critical signalling component for nuclear factor kappa B (NF-κB) activation and is also necessary for the production of many pro-inflammatory cytokines. In a mouse model with myeloid cell-specific deletion of Ikkβ an attenuated Kainic acid-induced hippocampal neuronal cell death was reported, suggesting a deleterious role of cytokines in this pathology (Cho et al. 2008). Identifying another microglia/macrophage-specific mechanism, the deletion of the NR1 subunit of the NMDA receptor in myeloid cells has been shown to attenuate neuronal damage in murine models of both traumatic brain injury and excitotoxicity (Kaindl et al. 2012). Further, microglial/macrophage deletion of p38α, a kinase critical to pro-inflammatory signalling, has been shown to attenuate neuroinflammation (Bachstetter et al. 2011), implicating microglia as a source of cytokines in the brain, and is neuroprotective for both lipopolysaccharide (LPS)-induced neuronal damage (Xing et al. 2011) and traumatic brain injury (Bachstetter et al. 2013). Glucocorticoid receptor deletion in macrophages and microglia also demonstrates enhanced neuro-inflammation and neuronal damage in a mouse model of PD (Ros-Bernal et al. 2011). Thus, together, in vivo and in vitro studies indicate that while microglia have the potential to actively initiate neuronal damage, and rather than framing microglia as a neurotoxic cell type, evidence supports that it is the dysregulation of only a handful of specific functions that results in microglia-mediated neuronal toxicity. Extensive research continues to explore the mechanisms shifting microglia to a neurotoxic phenotype.

6.4 Beneficial Microglia Responses

Various neuroprotective effects of microglia have been demonstrated in vivo in various experimental stroke paradigms. Microglia were found beneficial in ischemia in neonates (Faustino et al. 2011) and adults (Simard et al. 2006; Lalancette-Hebert et al. 2007; Yanagisawa et al. 2008; Cipriani et al. 2011), as well as in experimental stroke-like conditions in organotypic hippocampal slice cultures (OHSCs) (Neumann et al. 2006; Vinet et al. 2012). Transplantation of exogenous microglia into rodents (intraventricular or intra-arterial) before (Kitamura et al. 2005; Hayashi et al. 2006) or after (Neumann et al. 2006; Imai et al. 2007; Narantuya et al. 2010) experimental stroke reduced neural damage and improved neurological outcome of the animals. The beneficial response of microglia is not only limited to stroke conditions, since these cells were also found to be protective in other brain diseases in which neurons suffer from excitotoxicity such as Huntington's disease or traumatic brain injury (Simard and Rivest 2006; Palazuelos et al. 2009).

The range of potential neuroprotective mediators that are released from microglia in the ischemic brain is wide as there is evidence that these cells express neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glia cell-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) (Madinier et al. 2009; Narantuya et al. 2010), or release protective adenosine (Cipriani et al. 2011). Others have described that the pro-inflammatory cytokine TNFα in microglia acts as neuroprotective agent in stroke conditions (Sriram et al. 2006; Lambertsen et al. 2009) or provide more general evidence for protective role of microglia-related inflammation (Simard and Rivest 2006; Anrather et al. 2011). However, the role of inflammation in stroke is manifold. TNFα for example was found to be protective in the ischemic hippocampus, but promoted neuronal loss in the striatum in response to stroke (Sriram et al. 2006). Similarly most inflammatory mediators have double edged functions (protective or detrimental) in stroke showing that the overall outcome of their actions depends on a variety of conditions like temporary aspects (at what time point during the disease course) or spatial aspects (where is a cytokine released) (Iadecola and Anrather 2011). To unravel the protective side of microglia activity in excitotoxicity will be a major challenge for neuroscientists in the future, as these cells may be valid drug targets to prevent or treat brain diseases in which neurons suffer from being overexcited.

6.5 Loss of Microglial Function: Neuropathology

The loss of microglia function can experimentally be investigated in mouse models in which microglia are mutated, or in models that allow the specific inhibition or depletion of microglia.

6.5.1 Mutating Microglia Function

It is clear today that microglia in the brain are under constant restraint, particularly because they specifically express receptors for a variety of inhibitory factors that are constitutively expressed in the brain, mostly by neurons (Biber et al. 2007; Ransohoff and Perry 2009). The most prominent ligand-receptor pairs in this respect are CX3-chemokine ligand1-CX3-chemokine receptor1 (CX3CL1-CX3CR1) and OX-2 membrane glycoprotein (also called cluster of differentiation 200) and its receptor (CD200-CD200R), and mutations of these ligand-receptors pairs in mice have revealed much about microglia. Regarding the CX3CR1-CX3CL1 axis, one of the most used mouse model in microglia research is the CX3CR1-EGFP mouse line in which all microglia are GFP-positive (Jung et al. 2000). The consequences of CX3CR1 deletion in microglia largely depends on the mouse model used (see for extensive review: Prinz and Mildner 2011; Ransohoff and Prinz 2013; Wolf et al. 2013). However, the overall idea at the moment is that a lack of CX3CR1 leads to microglia "hyperactivity" in the diseased brain, thereby unleashing potential neurotoxic properties (Wolf et al. 2013). Accordingly, administration of CX3CL1 into the brain causes neuroprotection in experimental stroke and two models of PD (Cipriani et al. 2011; Pabon et al. 2011; Morganti et al. 2012). Similarly, removing the inhibitory input that is normally modulated by CD200 (i.e., as in CD200R knockout mice) reportedly promotes microglial morphological transition even in the healthy brain (Hoek et al. 2000) and leads to an exaggerated disease course both in experimental autoimmune encephalomyelitis (EAE) (an animal model of Multiple Sclerosis) (Broderick et al. 2002) and retinal inflammation (Hoek et al. 2000).

In ALS, mutations within the ubiquitously expressed enzyme superoxide dismutase 1 (SOD1) gene are responsible for about a quarter of the inherited disease cases. Accordingly, mice that express mutant human SOD1 exhibit motor neuron degeneration and a decreased life span (see for review: Lobsiger et al. 2009). The role of microglia in this disease has been investigated in various elegant experiments in which mutated SOD1 was expressed in specific cell types. The conclusion that arose from these experiments is that microglia with mutated SOD1 do not initiate motor neuron degeneration but rather accelerate disease progression (Xiao et al. 2007) (see for review: Lobsiger et al. 2009). The replacement of SOD1 mutated microglia with wild-type cells slowed down disease progression and prolonged the life span of the animals (Beers et al. 2006), which required functional myeloid differentiation primary response 88 (MyD88) signaling in microglia (Kang and Rivest 2007). MyD88 is an intracellular adapter protein that is required for the signaling of various pro-inflammatory receptors. Triggering receptor expressed on myeloid cells 2 (TREM2) is another receptor that is in brain exclusively expressed in microglia (for review see: Linnartz et al. 2010). TREM2 belongs to the family of immunoreceptor tyrosine-based activation motif (ITAM) receptors for which the ligand has yet not been identified. Activation of TREM2 stimulates phagocytic activity in microglia and downregulates TNFα and iNOS expression (Takahashi et al. 2005). TREM2 is thus an anti-inflammatory receptor that at the same time promotes

phagocytic activity. TREM2 is intracellularly coupled to the adapter protein DAP12 (for review see: Linnartz et al. 2010), and interestingly, loss of function mutations of either TREM2 or DAP12 lead to a rare chronic neurodegenerative disease known as Nasu-Hakola or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) (Colonna 2003). From the above description, it can thus be concluded that mutations in microglia mostly lead to or enhance the severity of a given brain disease or model.

6.5.2 Models of Microglial Inhibition or Depletion

6.5.2.1 Microglia Depletion with Clodronate

The bisphosphonate drug clodronate is toxic to cells of the myeloid lineage and can be used to selectively deplete them in vivo and in vitro (Buiting and Van Rooijen 1994). Since microglia are of myeloid origin, clodronate can also be used to deplete microglia in cell culture, OHSC, and in vivo (Kohl et al. 2003; Lauro et al. 2010; Drabek et al. 2012). OHSC was used to address the function of microglia in NMDA-induced neuronal loss by depleting resident microglia and then replenishing them with ectopic microglia (Vinet et al. 2012). It was found that neuronal cell loss was prominently increased in the absence of microglia (Vinet et al. 2012). It was also shown that when microglia-free OHSCs were replenished with microglia, these cells invaded the tissue, distributed themselves evenly across the slice, and acquired an in vivo-like, ramified morphology (Vinet et al. 2012). Most importantly, neurons in the presence of these ectopic microglia were protected from NMDA-induced toxicity to the same extent as in non-depleted control slices (Vinet et al. 2012). These findings convincingly show not only that microglia have a neuroprotective capacity, but also that this property applies to ramified microglia (Vinet et al. 2012). Thus, neurons are protected in the vicinity of ramified microglia, while removing microglia from the local environment renders neurons more vulnerable to excitotoxicity.

6.5.2.2 CD11b HSVTK Mouse Lines

Another way to specifically target microglia is through the use of transgenic mouse strains in which the herpes simplex virus thymidine kinase (HSVTK) is placed under the control of the CD11b promoter, which is active in most cells of myeloid origin (Heppner et al. 2005; Gowing et al. 2006). Application of gancyclovir to CD11b-HSVTK animals is mostly toxic to proliferating CD11b+ cells (Heppner et al. 2005; Gowing et al. 2006). The effects of gancyclovir on microglia in vivo are dependent on the application route of the drug in these animals. If peripheral gancyclovir application (intraperitoneal injection or oral application) is used, transplantation of wild-type bone marrow is required to spare the peripheral myeloid

compartment from gancyclovir treatment. In these resulting chimeric animals, gancyclovir application leads to the inhibition of morphological microglia transition to amoeboid cells in the case of EAE (referred to as microglia paralysis (Heppner et al. 2005)) or to the death of microglia undergoing proliferation after experimental stroke (Lalancette-Hebert et al. 2007). Whereas the inhibition of morphological microglia transition (microglia paralysis) was protective in EAE (delayed disease onset and reduced clinical scores) (Heppner et al. 2005), the ablation of microglia proliferation in the stroke model led to a larger stroke lesion area and increased neuronal death (Lalancette-Hebert et al. 2007).

More recent studies using this mouse line have changed the application route of gancyclovir from peripheral to central, which specifically depletes the treated brain tissue of ramified microglia without affecting the peripheral myeloid cells (Gowing et al. 2006; Grathwohl et al. 2009; Mirrione et al. 2010; Varvel et al. 2012). In the corresponding studies it was shown that the depletion of microglia by gancyclovir did not affect the development of amyloid-beta (Aβ) plaques in two different mouse models of AD (Grathwohl et al. 2009) nor did the absence of microglia changed disease progression and motor neuron degeneration in the SOD mouse model of ALS (Gowing et al. 2008). However, in the case of pilocarpine-induced seizures, the depletion of microglia prevented the seizure-reducing effect of LPS pre-conditioning, indicating that the inflammatory capacity of microglia was beneficial in this mouse model (Mirrione et al. 2010). Taken together, it can be concluded that gancyclovir-dependent inhibition of microglia function in CD11b-HSVTK animals is only advantageous in one disease model, which is EAE (Heppner et al. 2005). All other reports either provided evidence for a beneficial role of microglial function in vivo (Lalancette-Hebert et al. 2007; Mirrione et al. 2010) or showed no effect of blunting the microglial response (Gowing et al. 2008). It should be noted here that the latter studies inhibited or depleted microglia for a limited time, and at rather late stages of chronic disease models (Gowing et al. 2008; Grathwohl et al. 2009), which may explain the surprising lack of effect. The inhibition or depletion of microglial function may have been too late or too short to unravel the role of these cells in mouse models of AD and ALS (Gowing et al. 2008; Grathwohl et al. 2009). Thus, inhibition or depletion of microglia for longer time periods may be required for chronic disease models.

Taken together, mutating or depleting microglia is rarely correlated to an improved outcome in various brain disease models. These findings together with the discussed reports about the consequences of mutating microglia in the brain clearly argue for a protective role of the innate immune cells of the brain.

6.6 Regulating Microglia Responses

Current theories on microglia-mediated neuronal damage holds that environmental compounds (Taetzsch and Block 2013), neuronal damage (Gao et al. 2003b; Perry and Teeling 2013), aging (Norden and Godbout 2013; Perry and Teeling 2013), and

CNS diseases (Perry and Teeling 2013) may prime microglia to be overly sensitive to stimuli, thus promoting an exaggerated (Ransohoff and Cardona 2010; Xiong and Kielian 2013) and chronic (Ransohoff and Cardona 2010; Gomez-Nicola et al. 2013) response to exert toxicity.

As described above there have recently been tremendous changes in our understanding of microglia "activation", yet different states (or phenotypes) of microglial responses have not been clearly defined so far. This is different from macrophage biology where the response can be classified as an M1 (classical/pro-inflammatory activation), M2a (alternative activation/anti-inflammatory activation), and M2c (deactivation/wound healing activation) response (Ginhoux et al. 2010; Boche et al. 2013; Jang et al. 2013). M1 activation is characterized by the upregulation of inflammatory mediators (ex. TNFα, IL-1β, COX2, and iNOS) and the production of ROS (ex., H_2O_2, peroxynitrite ($ONOO^-$)) (Block et al. 2007). Critical for the regulation of the immune response, the initial M1 response is typically followed by a secondary M2 activation that is important for wound healing and resolving inflammation, which is marked by the expression of factors such as Arginase1 (AR1, decreased iNOS activity), chitinase 3-like 3 (Ym1, tissue remodeling), and Found in Inflammatory Zone 1 (Fzz1, tissue remodeling) (Jung et al. 2000; Ginhoux et al. 2010; Boche et al. 2013). Microglia, being of myeloid origin, can express M1/M2 markers that might provide some insight into their phenotype. However, microglia are highly plastic cells that may rapidly transit between different states (or phenotypes) and it is at the moment controversially debated whether or not the M1/M2 classification also is valid in the microglia field. Here, we will use the terms M1-like and M2-like as descriptions for marker patterns that resemble the markers expressed by M1/M2 macrophages. However, these terms are not used as descriptors of function; the actual impact of the microglial response on neuronal survival will depend on timing, the degree of the response, the chronic nature of the response, and may very well be model/disease-specific.

6.7 The Excessive and Chronic Microglial Response

Microglial immune responses to pathogens, environmental toxins, and neuronal damage join the long list of beneficial responses that microglia perform to promote CNS health in the case of normal physiology (Varnum and Ikezu 2012). However, accumulating evidence indicates that a deleterious and neurotoxic microglial phenotype occurs when microglia activation is dysregulated to become a polarized M1-like phenotype (Hu et al. 2012; Bechade et al. 2013), which is defined by an exaggerated pro-inflammatory response (M1-like) with impaired resolution (M2-like response) (Aguzzi et al. 2013; Jang et al. 2013). Thus, while microglia expressing M2-like markers are associated with repair such as remyelination (Miron et al. 2013) and desensitization to pro-inflammatory stimuli (Ajmone-Cat et al. 2013), microglia with M1-like markers are implicated

in chronic neuroinflammation and progressive neuron damage (Hu et al. 2012; Ardeljan and Chan 2013; Blandini 2013). As such, current research is focusing on mechanisms and techniques capable of shifting microglia to a polarized M2-like phenotype for neuroprotection (Zhang et al. 2013).

6.8 Triggers of the Microglial M1-Like Expression Pattern

At present, there is also considerable interest in identifying the circumstances instigating the pathological microglia response.

6.8.1 Immune Stimuli

There is a wealth of evidence that microglia detect and respond to paracrine and autocrine pro-inflammatory signals implicated in M1 polarization, such as TNFα, ROS, ATP, IL-1β, chemokines, etc. (Hanamsagar et al. 2011; Harms et al. 2012). Interestingly, aging (Michaud et al. 2013) and neurodegenerative diseases such as PD (Reale et al. 2009) and AD (Michaud et al. 2013) have been linked with peripheral immune dysregulation, where pro-inflammatory markers are elevated in the blood. For example, pro-inflammatrory cytokines are elevated in PD patient blood (Chen et al. 2008) and upregulated in the circulating white blood cells, both at basal levels and in response to LPS (Reale et al. 2009), indicating that these peripheral immune cells are biologically altered during the process of CNS pathology. Peripheral inflammation is documented to transfer to the brain in adult animals (Qin et al. 2007) and in utero (Carvey et al. 2003) to activate microglia through circulating cytokines. Importantly, the peripheral immune response has been documented to shift the neuroinflammatory phenotype in the brain, modulating and priming CNS macrophages to be more sensitive to additional pro-inflammatory stimuli (Perry 2004; Puntener et al. 2012). In addition to systemic bacterial infection/cytokines, microglia are also able to detect and respond to other forms of peripheral pathology that elevate circulating cytokines, such as kidney damage (Liu et al. 2008) and intestinal reperfusion injury (Hsieh et al. 2011), posing the interesting premise that microglia may survey and be reprogrammed by peripheral disease/damage.

6.8.2 Neuronal Damage

Critical for wound healing, the microglial response to CNS injury or neuron damage (i.e., reactive microgliosis) was initially perceived as only a transient event (Streit et al. 1999). However, current views hold that this response has the potential to be

both long-lived and self-propagating (Gao et al. 2003a; Huh et al. 2003; McGeer et al. 2003). In normal physiology, particularly in the case of apoptosis, neuronal death should not promote M1-like polarization in microglia (Minghetti et al. 2005). However, in disease and pathology, reactive microgliosis is often documented being M1-like and has been implicated as a toxic component of many neurodegenerative diseases (see below for examples) (Eikelenboom et al. 2002; Wenk 2003; Sanchez-Moreno et al. 2004).

One key proposed mechanism through which dying neurons are believed to promote the M1-like response in microglia is the loss of neuron–microglia interactions. Thus, removal of inhibition is a discussed component of initiation of the microglial M1-like response in vivo, where neurons routinely provide many of these anti-inflammatory signals for homeostatic maintenance of the microglial phenotype in normal physiology (Ransohoff and Cardona 2010). More specifically, the disruption of anti-inflammatory cell–cell interactions such as CD200-CD200R-mediated (Hoek et al. 2000) in addition to the depletion of inhibitory-soluble ligands released by damaged neurons, such as CX3CL1 or fractalkine (Suzumura 2013), may set the stage for neurotoxic reactive microgliosis (see below for more detailed information). Furthermore, neuron injury signals released by damaged neurons that can either initiate or amplify the M1-like response have also been identified, including, matrix metalloproteinase-3 (MMP-3) (Kim et al. in review), α synuclein (Zhang et al. in press), μ calpain (Levesque et al. 2010), neuromelanin (Wilms et al. 2003; Zecca et al. 2003), ATP/UTP (Domercq et al. 2013), oxidized mSOD1 protein (Appel et al. 2011), glutamate (Domercq et al. 2013), extracellular nucleotides (Domercq et al. 2013), zinc (Kauppinen et al. 2008), and Heat Shock Protein 60 (HSP60) (Zhang et al. 2012). Together, it is both the removal of inhibitory signals essential in neuron-microglia communication and the release of M1 triggers from damaged neurons that interact to culminate in chronic dysregulation of the microglia in response to neuronal injury.

6.8.3   Endogenous Disease Proteins

Several hallmark proteins from neurodegenerative diseases and CNS pathology also directly activate the microglial M1-like response (see further information in Chap. 18). In the case of AD, the Amyloid Hypothesis postulates that Aβ has a causative role in AD pathology, which may occur through toxicity directly to neurons (Yankner 1989; Yankner et al. 1990) and microglia-mediated neuron damage (Combs et al. 2000; Qin et al. 2002). It is well known that microglia migrate to and cluster around senile plaques containing Aβ and neurofibrillary tangles (McGeer et al. 1987; Sasaki et al. 1997), change morphology (Meda et al. 1995), and produce pro-inflammatory factors, such as nitric oxide (Li et al. 1996), superoxide (Qin et al. 2002; Wilkinson and Landreth 2006), and TNFα (Dheen et al. 2004) in response to Aβ.

α Synuclein is another example of a disease protein that is known to directly interact with microglia to elicit an M1-like expression pattern. α Synuclein is a component of Lewy Bodies, a hallmark feature of PD pathology (Goedert et al. 2013; Trinh and Farrer 2013). Levels of the α Synuclein protein are elevated in the midbrain of sporadic PD patients (Chiba-Falek et al. 2006; Shi et al. 2011) and over-expression of wild-type α Synuclein (SNCA) due to genetic multiplication causes early onset, autosomal dominant-familial PD (Sironi et al. 2010), implicating the protein in PD. Elevation of α Synuclein occurs early in PD progression and, as such, has been proposed as a preclinical marker of PD (Chahine and Stern 2011; Shi et al. 2011). Importantly, the increase in microglial markers in the PD SNpc is linked to the degree of α Synuclein deposition (Croisier et al. 2005). Both the α synuclein monomer (Lee et al. 2010) and the aggregated (Zhang et al. 2005, 2007; Lee et al. 2010) forms have been shown to cause the production of pro-inflammatory cytokines (TNFα and IL-1β), ROS, and microglia-mediated neurotoxicity in vitro.

6.8.4 Pesticides

Several pesticides are linked to an increased incidence to PD and a direct interaction with M1-like polarization of microglia. Paraquat (*N,N*′-dimethyl-4,4′-bipyridinium dichloride) is an extensively used herbicide associated with increased PD risk (Hertzman et al. 1990; Liou et al. 1997; Costello et al. 2009; Wang et al. 2011). Paraquat is believed to cross the blood-brain barrier through the neutral amino acid transporter following systemic exposure (Shimizu et al. 2001; Chanyachukul et al. 2004), thereby causing neuroinflammation (Mitra et al. 2011; Mangano et al. 2012) and selective toxicity to DA neurons of the SNpc (McCormack et al. 2002) in vivo (Fei et al. 2008). In vitro data suggest that while paraquat directly damages neurons at high concentrations (Richardson et al. 2005), lower concentrations are thought to be neurotoxic predominantly through microglial activation (Wu et al. 2005). A single exposure to paraquat in mice causes changes in microglia morphology in the SNpc, thus indicating immune perturbation, where subsequent exposures to either the pesticide paraquat (Purisai et al. 2007), the fungicide maneb (Cicchetti et al. 2005), or LPS (Purisai et al. 2007) results in enhancement of the pro-inflammatory response. In vitro studies in primary cultures have shown that microglia treated with paraquat do not initiate the full M1-like response, but rather, they only produce ROS (Bonneh-Barkay et al. 2005; Wu et al. 2005; Miller et al. 2007). As such, inhibition of iNOS (Yadav et al. 2012) and NOX2 (Taetzsch and Block 2013), as well as co-treatment with a superoxide dismutase mimetic (Peng et al. 2009), decreases the microglial response to paraquat. Exposure to the pesticides rotenone, dieldrin, and lindane is also associated with an increased risk for PD (Dhillon et al. 2008; Tanner et al. 2011; Weisskopf et al. 2010; Mao and Liu 2008), where activated microglia have been shown to produce extracellular superoxide (Mao and Liu 2008; Zhou et al. 2012). Lindane, dieldrin, paraquat, and rotenone also activate a partial M1-like microglial response with ROS production (Taetzsch and Block 2013), but the mechanisms of action are poorly understood.

6.8.5 Air Pollution

While a chronic microglial M1-like response is implicated in pathology, recent reports indicate that microglia are capable of detecting exposure to various forms of air pollution, which may arguably be one of the most common environmental sources of microglial M1-like activation (Block et al. 2012). Air pollution is a complex mixture derived from numerous natural and anthropogenic sources, including particulate matter (PM); ozone, carbon sulfur oxides, nitrogen oxides, methane, and other gases, volatile organic compounds (e.g. benzene, toluene, and xylene), and metals (e.g. lead, manganese, vanadium, iron) (Block et al. 2012). Elevated exposure to air pollution is linked to CNS diseases, behaviour deficits, neuroinflammation, and neuropathology in human and animal studies (Block and Calderon-Garciduenas 2009; Lucchini et al. 2012). Imaging of postmortem human brain tissue has even identified the particle components of urban air pollution in the brain parenchyma (Calderon-Garciduenas et al. 2008). Animal studies have shown that exposure to ambient PM (Campbell et al. 2005, 2009), ozone (Santiago-Lopez et al. 2010), diesel exhaust (Gerlofs-Nijland et al. 2010; Levesque et al. 2011a, b), and manganese (Elder et al. 2006; Antonini et al. 2009) results in elevated cytokine expression and oxidative stress in the brain. Importantly, analysis of postmortem human samples links air pollution to a M1-like microglial response in humans (Calderon-Garciduenas et al. 2008) and animal studies (Levesque et al. 2011b; Morgan et al. 2011; Bolton et al. 2012). Current studies are centred on understanding the particular contexts where this microglial response impacts neuron survival.

6.9 Triggers of the Microglial M2-Like Expression Pattern

Since microglia expressing M2-like patterns are currently seen as beneficial elements, it would be desirable to gain more information about the signals that initiate M2-like patterns in microglia. Little is known about the triggers that lead to microglial M2-like expression patterns. In macrophages, the cytokine Interleukin-4 (IL-4) is often used as a stimulus for M2 polarization, and it was shown some years ago that IL-4 induces cultured microglia to promote oligodendrogenesis (Butovsky et al. 2006). A year later it was described from the same group that IL-4-treated microglia express some dendritic cell-like markers (Butovsky et al. 2007), but whether or not IL-4 causes a M2-like microglia pattern was not investigated. Data in favour of this assumption were published by Ponomarev and colleagues. Using knockout animals and bone-marrow chimeras they showed that IL-4 in the CNS but not in the periphery is required to cause a M2-like microglia response, correlated in that case to diminished EAE disease course (Ponomarev et al. 2007). In aged mice it was found that ex vivo microglia are less sensitive to IL-4 treatment compared to microglia derived from young adult mice (Fenn et al. 2012), suggesting that aging reduces the protective properties of this cytokine. In this regard it is interesting to

note that aging is associated with an M1-like pro-inflammatory response in microglia (for further details see Chap. 13). Recently, it was found that the microglia cell line BV-2, when treated with IL-4, express some major M2 markers and are found to be neuroprotective in OHSCs subjected to oxygen-glucose deprivation (Girard et al. 2013). It should be noted here, however, that untreated BV2 cells in the same paradigm display more pronounced neuroprotective properties than IL-4 treated cells (Girard et al. 2013) in agreement with earlier findings (Neumann et al. 2006).

Interestingly, various lines of evidence suggest that chronic treatment with low doses of LPS causes an M2-like pattern in brain microglia, which might be the potential mechanism for the known LPS-dependent preconditioning (Chen et al. 2012; Ajmone-Cat et al. 2013). The potential therapeutic value of LPS-dependent preconditioning, however, remains to be established. In conclusion, while microglia express many M2 markers during different functions, it is unlikely that microglia follow a strict macrophage activation pattern typical of peripheral myeloid cells. Significant study is needed to resolve the role of M2-like activation in microglial function and phenotype.

6.10   Implications

Microglia are the myeloid cells of the CNS. They are derived from a specific embryonic myeloid cell population and these cells invade the developing brain very early. They become true brain cells in a sense as they do not leave the brain and are a self-renewable population that is not replaced by peripheral myeloid cells. Microglia are protective elements in the brain, because mutating or deleting them is seldom associated with a beneficial outcome in an acute injury situation. This however might change in chronic disease or the aged brain. The chronic exposure to a variety of stimuli may lead to the development of microglia with a M1-like expression pattern; aging may cause similar processes. These often called "primed" microglia may initiate or participate in neurodegenerative diseases, thus turning the original beneficial phenotype of microglia into a potentially neurotoxic one. It will be a major challenge for future research to understand the molecular mechanisms that trigger the development of neurotoxic microglia.

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